AD-A084 700 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
PLASMID DETECTION IN LEGIONELLA PNEUMOPHILA AND LEGIONELLA-LIKE--ETC(U)
APR 80 P MIKESELL, J W EZZELL, G B KNUDSON

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1 OF 1
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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
	AD-A084 700	

4. TITLE (and Subtitle)
PLASMID DETECTION IN LEGIONELLA PNEUMOPHILA AND LEGIONELLA-LIKE ORGANISMS

5. TYPE OF REPORT & PERIOD COVERED
Interim

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
PERRY MIKESELL,* J. W. EZZELL AND GREGORY B. KNUDSON

8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS
U.S. Army Medical Research Institute of Infectious Disease SGRD-UIB-A
Fort Detrick, Frederick, MD 21701

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
LPN USAMRIID
A91C-00-133

11. CONTROLLING OFFICE NAME AND ADDRESS
U.S. Army Medical Research and Development Command, Office of the Surgeon General
Department of the Army, Washington, D.C. 20314

12. REPORT DATE
30 April 1980

13. NUMBER OF PAGES
13 pages

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)
UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited.

DTIC ELECTE MAY 1980

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES
Information regarding reprints not available at this time. It has been submitted for publication in Infection and Immunity.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Plasmid, Legionella pneumophila, Legionella-like organisms

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
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DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

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(11) 30 April 1980

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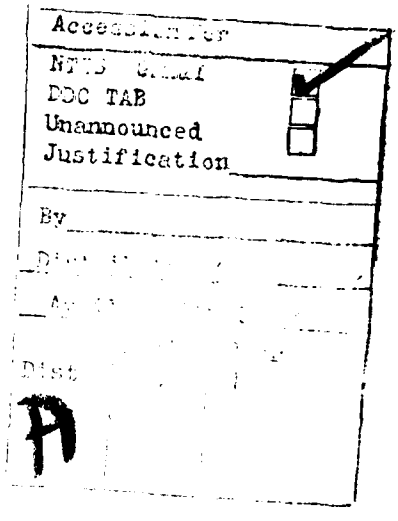

Approximately one third of all diagnosed cases of pneumonia in the United States each year cannot be attributed to known viral or bacterial agents (7). Recent evidence has demonstrated that certain strains of bacteria, designated Legionella-like organisms, can cause pneumonia in man (3, 14, 17). These bacteria, like many other medically important bacteria, may contain plasmid-mediated drug resistance or virulence factors (20). The demonstration of either of such factors could alter the chemotherapeutic management of patients with diseases caused by these organisms. This paper describes the isolation of plasmid deoxyribonucleic acid (DNA) from three of the six isolates tested.

All strains examined, WIGA, HEBA, TATLOCK, OLDA, PPA and TEX-KL, possess varying degrees of phenotypic or genotypic relatedness to L. pneumophila. The "Pittsburgh Pneumonia Agent" (PPA), (18) and the HEBA and TATLOCK bacteria (10) are believed to be members of a bacterial species separate from L. pneumophila (11). The OLDA bacterium, originally isolated in 1947, is now considered to be a strain of L. pneumophila, serogroup 1 (15). Two additional agents, TEX-KL and WIGA, have been proposed to represent a bacterial species, phenotypically similar but genetically unrelated to Legionella (14). Hereafter, all isolates will be referred to as Legionella-like organisms, recognizing that the OLDA isolate is a strain of L. pneumophila.

Bacteria were cultured on charcoal yeast extract agar (6) or in yeast extract broth (19) according to established parameters of growth for Legionella. Plasmids were detected utilizing agarose gel electrophoresis (16). While there are numerous modifications of this technique, most require some measure of plasmid purification prior to electrophoresis. One variation of the procedure is to lyse intact cells directly in the agarose wells (1). We have used a similar method to

screen Legionella-like bacteria for plasmids (5). One or two colonies from plates are resuspended directly in an agarose well containing 15 µl of lysozyme mixture [lysozyme, 0.2 mg/ml; ribonuclease I, 0.1 µg/ml; 0.05% bromphenol blue and 20% Ficoll 400,000 in Tris-borate buffer (89mM Tris base, 2.5 mM disodium EDTA and 8.9 mM boric acid, pH 8.2)]. This cell suspension was allowed to stand at room temperature for at least 15 min to assure spheroplast formation. Thirty µl of lysis mixture [0.2% sodium dodecyl sulfate (SDS) and 10% Ficoll 400,000 in Tris-borate buffer) were added to each well, mixed gently with a toothpick and overlaid with 100 µl of 0.2% SDS and 5% Ficoll 400,000 in same buffer and sealed with molten agarose. The definition of plasmid bands was improved if cells from broth cultures or plates were first resuspended in 20% Ficoll 400,000 in Tris-borate buffer before being added to the lysozyme mixture in the well. This observation may reflect inadequate mixing of cells in the well when samples are taken directly from solid media. Gel concentrations were varied from 0.7 to 1% agarose to enhance plasmid separation and define covalently closed circular and open circle forms of plasmid species. The samples were subjected to electrophoresis in Tris-borate buffer at 2 mA for 60 min followed by 50 mA for 100 to 180 min using a BioRad Model 220 electrophoresis cell and Model 500 power source. Following electrophoresis gels were stained with ethidium bromide (0.5 g/ml in distilled water) for 30 min followed by two 15-min rinses in distilled water. Plasmid bands were visualized using an ultraviolet transilluminator (Model C-63, Ultraviolet Products, San Gabriel, Calif).

Three of the six isolates examined, PPA, HEBA and TATLOCK, did not exhibit plasmid DNA. A single isolation or detection procedure may not be suitable for isolating plasmids from a given bacterial strain (13)

and alternate methodologies may eventually establish the presence of plasmid DNA in these bacteria. Pseudomonas aeruginosa was used as a molecular weight marker for all runs (12) (Fig. 1A). Plasmid DNA was detected in the WIGA, OLDA and TEX-KL isolates (Fig. 1B-D). These bands appear to represent separate plasmid species based on band migration patterns under different electrophoretic conditions. There also appear to be two bands of material migrating in the chromosome region (Fig. 1B-D). Resolution of these bands was obtained with the OLDA isolate by changing electrophoretic conditions (Fig. 2). Ostensibly, there are four distinct plasmids in the OLDA bacterium. A similar observation has not been made in either WIGA or TEX-KL and it is not yet known whether these two isolates also contain smaller molecular weight plasmids.

The method described above is economical, rapid and well suited to epidemiological survey work, although there are limitations. This is a screening procedure, and as such, definitive molecular and genetic characterizations of plasmid material are not possible. We believe the plasmids are large; therefore, estimates of plasmid molecular weight are not possible using this electrophoretic system (9). Attempts to purify the plasmids from these bacteria by established methods have been unsuccessful (2, 4, 8). The plasmids may be highly susceptible to shear by virtue of their size or they may exhibit some degree of nonintegrated association with the host chromosome, both factors complicating purification. It should also be emphasized that the cell concentration can greatly affect the detection of plasmid material particularly in the 12 to 19 megadalton range. We apply 15-, 30- and 40-µl amounts of sample material to the gel to preclude masking of plasmids which migrate near the chromosomal DNA. This is particularly advantageous if optimal growth conditions have not been defined for a given isolate.

We have established the presence of extrachromosomal genetic elements in two Legionella-like organisms, WIGA and TEX-KL, and in the OLDA strain of L. pneumophila. The results indicate that Legionella organisms are able to maintain plasmid elements as part of their genetic composition. The ability of these organisms to exchange genetic material with other bacteria has, to our knowledge, not been reported. However in view of the narrow spectrum of antibiotics effective in the treatment of legionellosis and Legionella-like diseases, the acquisition of drug resistance factors by the bacteria could have serious clinical ramifications.

ACKNOWLEDGMENT

We wish to thank G. A. Jacoby for supplying us with Pseudomonas aeruginosa, PU21. We are also indebted to Earnest L. Cook and Helen Hargett for their excellent technical assistance.

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FIGURE LEGENDS

FIG. 1. 1.0% agarose gel of plasmid DNA. Gels were run at 2 mA for 60 min followed by 50 mA for 150 min. Migration is from top to bottom. (A) P. aeruginosa, PU21 (12) (plasmid molecular weight 312 megadaltons). Smaller 20-megadalton plasmid is not evident. (B) TEX-KL, two distinct plasmid bands representing separate molecular species. (C) OLDA a single plasmid band is evident migrating in the same region as top band of TEX-KL. (D) WIGA, very faint band (arrow) migrates in the same region as lower band of TEX-KL. The large diffuse band in all wells is chromosome. Note double band appearance in chromosome region (B, C, and D).

FIG. 2. 0.7% agarose gel run of OLDA. Electrophoresis was carried out at 2 mA for 60 min followed by 50 mA for 105 min. There appear to be four separate plasmid bands. The two bands immediately below and the single band immediately above the chromosomal band are masked by the chromosomal DNA shown in Fig. 1C.